



Luminescence from lanthanide(III) ions bound to the Glycocalyx of Chinese Hamster Ovary cells

Arppe-Tabbara, Riikka; Carro-Temboury, Miguel R; Hempel, Casper; Vosch, Tom; Sørensen, Thomas Just

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Luminescence from lanthanide(III) ions bound to the Glycocalyx of Chinese Hamster Ovary cells

Riikka Arppe-Tabbara,^[a] Miguel R. Carro-Temboury,^[a] Casper Hempel,^{*[b]} Tom Vosch^{*[a]} and Thomas Just Sørensen^{*[a]}

Abstract: Lanthanide(III) ions bind to the glycocalyx of Chinese Hamster Ovary (CHO) cells and give rise to a unique luminescent fingerprint. Following direct excitation of terbium(III) or europium(III) ions in the visible part of the spectrum, we are able to collect emission spectra pixel-by-pixel in images of CHO cells. Following data analysis that removes the background signal, the fine structure of the europium(III) luminescence indicate that the lanthanide(III) ions are bound to a single structure of the CHO cell glycocalyx. This was deduced from the fact that the structure-sensitive emission spectrum of europium is unchanged throughout the investigated samples.

Lanthanide luminescence is unique as it manifests as distinct series of narrow emission lines with characteristic luminescence lifetimes.^[1] Either feature can be used to generate high contrast in fluorescence microscopy,^[2] which has been demonstrated for bioimaging using molecular probes based on green emitting terbium(III) or red emitting europium(III) ions.^[3] The probes are normally based on sensitized lanthanide luminescence,^[1, 3f] where antenna chromophores are used to negate the low absorption coefficients of the lanthanide ions (Figure 1). While the design principle has been expanded to more lanthanide(III) ions,^[4] it remains an unsolved challenge to create sensitizer chromophores that allow for excitation of lanthanide-based molecular probes using visible light. Here, we demonstrate that we can circumvent the sensitization step and use direct excitation of free europium(III) and terbium(III) ions and still record images and luminescence spectra from a biological sample by using a state-of-the-art fluorescence microscope.^[2a, 5] Furthermore, we show that we can obtain background subtracted luminescence spectra from the individual pixels. Thereby, we are able to show that lanthanide(III) ions give rise to identical spectra in all pixels where they are present, suggesting that the ions exclusively bind to selected components of the glycocalyx of CHO cells. Exploiting the unique luminescence features of lanthanides with narrow emission lines and long lifetimes was first demonstrated with labile complexes.^[2e, 6] Of note are the ultrasensitive DELFIA® assay and time-gated high-throughput screening platforms based on the long luminescence lifetime of several lanthanide(III) ions.^[6e, 7] Since the first demonstration, the focus has been on making molecular probes from kinetically inert complexes as they allow for clinical applications.^[2d, 3a, 3e, 8]

One drawback of using sensitized lanthanide luminescence is that lanthanides are excited in the ultraviolet part of the spectrum. To enable excitation in the visible part of the spectrum direct excitation of forbidden *f-f* transition can be used. Figure 1 shows the absorption spectra of a thioxanthone antenna chromophore and europium(III) and terbium(III) acetate. There is a 1000-fold difference in absorption efficiency which, however, is easily overcome by the efficiency of modern light sources and photodetectors—even spectrometers—with single molecule sensitivity.^[2a, 5]

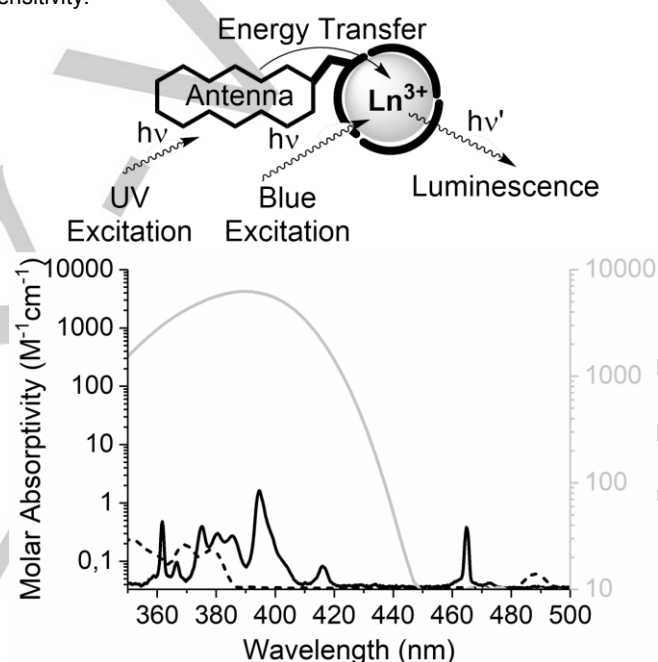


Figure 1. Top: Depiction of the antenna principle in lanthanide luminescence. Bottom: Absorption spectra of europium(III) acetate (black, solid) and terbium(III) acetate (black, dash) in water, and an aza-thioxanthone dye (light grey, right y-axis) in methanol.^[9]

While bioimaging following direct excitation of lanthanide ions has been demonstrated for kinetically inert lanthanide complexes,^[10] the use of free lanthanide(III) ion based probes for optical imaging has not been reported. This is not the case in bioimaging using transmission electron microscopy (TEM),^[11] where lanthanide(III) ions are well-known contrast agents. In TEM-based bioimaging, several protocols based on lanthanide(III) ions have been used for selective staining of glycocalyx.^[11a, 11b, 12] Optical bioimaging of glycocalyx primarily relies on specific antibodies or lectins that are detected with fluorochromes.^[11b, 13] Here, we show that the lanthanide(III) ions that provide contrast in TEM, upon direct excitation can generate sufficient contrast in a confocal fluorescence microscope for luminescence spectra to be recorded in the individual 1 by 1 micrometer pixel.

[a] Dr R. Arppe-Tabbara, Dr M.R. Carro-Temboury, Dr T. Vosch, Dr T. J. Sørensen

Nano-Science Center & Department of Chemistry
University of Copenhagen
Universitetsparken 5, 2100 København Ø, Denmark
E-mail: tom@chem.ku.dk, tjs@chem.ku.dk

[b] Dr C Hempel
Department of Micro- and Nanotechnology
Technical University of Denmark
Kgs Lyngby, Denmark
E-mail: casperhempel@gmail.com

We chose to image formaldehyde fixed CHO cells following live cell staining (see Supporting Information for details). The cells were grown on cover slips and stained with a solution containing either 40 mM lanthanide(III) acetate or a cocktail of lanthanide(III) acetate, F18, and Syto-9. The cells were then fixed with formaldehyde and then treated with SlowFade Gold antifade mounting medium, and mounted on a microscopy slide. Samples were prepared with terbium(III) acetate and europium(III) acetate. Spectrally resolved images were recorded of the four different samples types on a home-built confocal fluorescence microscope with single molecule detection capabilities.^[2a, 5]

All samples were imaged in a 60-by-60-pixel array with a pixel size slightly exceeding 1 by 1 micrometer. To achieve sufficient signal a 1 s integration time per pixel was used, note that even in a state-of-the-art microscope acquiring these images takes up to 1 hour. The spectrally resolved images show that lanthanide(III) ions localize to the glycocalyx, as the spectra obtained from different regions of CHO cells only show the distinct fingerprint of lanthanide luminescence in the intercellular space. Figure 2 shows a representative image of a CHO cell stained with europium(III) acetate, F18 (a green membrane stain), and Syto-9 (a green nucleus stain) imaged following 465 nm excitation. F18 and Syto-9 allows the membrane and nucleus to be identified and spectra from individual pixels in selected regions of the cell are included in Figure 2. It is of note that no lanthanide(III) emission is seen inside the cells. The europium(III) emission spectra from the pixels containing europium(III) ions in Figure 2 are all similar. As the shape and relative peak ratios of the europium(III) spectrum depends on the symmetry and coordination chemistry,^[1, 14] it will be different when in solution compared to when bound to the glycocalyx.

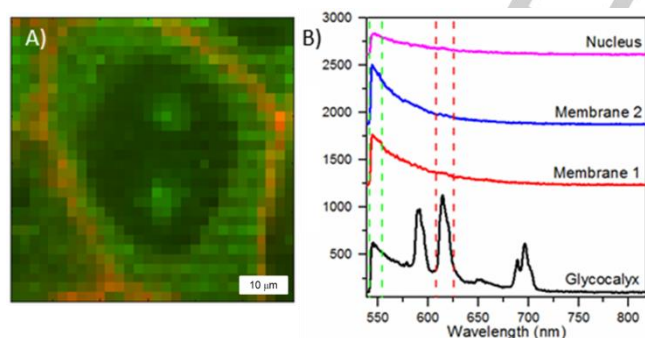


Figure 2. CHO cells stained with europium(III) acetate, F18 and Syto-9 imaged in a 60 by 60 micrometer area using a confocal scanning microscope equipped with a CCD spectrometer following 465 nm excitation. A, Pseudo-colored image, B, spectra from individual pixels from selected regions in the images corresponding to the cell nucleus, membrane and glycocalyx. The green and red dashed lines in the spectra illustrate the spectral regions used for pseudocoloring.

To investigate the sharp lines resulting from lanthanide luminescence in detail, we chose to resolve these using our previously introduced image analysis.^[15] This allows the fluorescent signal arising from lanthanide centered emission to be fully distinguished from auto-fluorescence and the signal

arising from other fluorescent dyes. Figure 3 shows an image of CHO cells stained with europium(III), F18, and Syto-9. As expected, the two green-emitting dyes and the red emitting lanthanide(III) ion are excited at 465 nm, and give rise to localized emission from the glycocalyx, membrane and nucleus, respectively. By plotting a pseudo-color image (Figure 3A) the individual cell components can be identified, even though probes cannot be differentiated. Figure 3A shows that the membrane, the nuclei and the intercellular space are stained, and that the cytosol is dark. As we are able to uniquely identify the photons arising from europium(III) centered emission,^[15] we can conclude that the lanthanide luminescence gives rise to the orange-red areas in Figure 3A. The europium emission lines are clearly resolved in the total emission spectrum (from all pixels, Figure 3B). Image analysis allows us to resolve the counts originating from lanthanide centered emission, which gives rise to the background free image shown in Figure 3C that clearly shows that the europium(III) ions are localized in the extracellular space. This further allows the europium(III) emission spectrum to be studied in detail, as evident in the total emission spectrum shown in Figure 3D.

Figure 4 shows an image of CHO cells stained with terbium(III) acetate. The lanthanide(III) ions are excited directly at 488 nm, and gives rise to localized emission from the glycocalyx. By plotting a pseudo-color image (Figure 4A) it is evident that contributions from auto-fluorescence originating from the cell plasma membrane give rise to contrast as well. In Figure 4B the narrow emission lines originating from terbium centered emission and a broad background signal from auto-fluorescence are shown. Using the data analysis algorithm pixel-by-pixel to uniquely identify the photons arising from terbium(III)-centered emission we obtain the image and spectrum shown in Figure 4C and 4D.^[15] cursory inspection of the treated image shows that only the intercellular space contains terbium(III) ions. The emission spectra in Figure 4B and 4D, from all pixels in the images, show that the lanthanide emission lines are resolved with a perfect contrast.^[15] Note that of the three peaks visible in the emission spectrum recorded over the entire spectrum (Figure 4B), only the most intense one is so intense that it is resolved by the algorithm that resolves the signal pixel-by-pixel.^[15] Thus, the images in Figure 2, 3 and 4 suggest that lanthanide(III) ions are found exclusively in the intercellular space and most likely in the glycocalyx of the CHO cells. Further examples can be found in the supporting information.

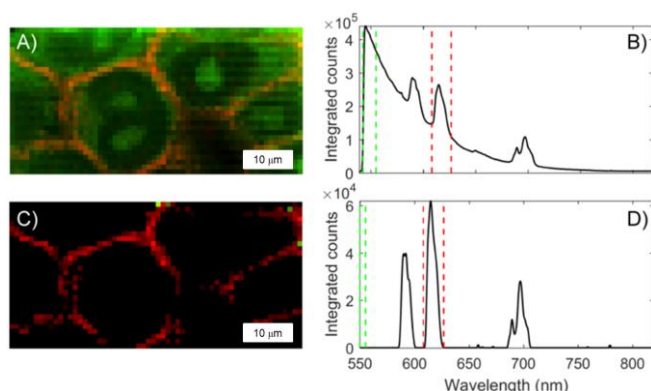


Figure 3. 30 by 60 micrometer image of a single region of CHO cells stained with europium(III) acetate, F18 and Syto-9 imaged in a confocal scanning microscope equipped with a CCD spectrometer and following 465 nm excitation. A, pseudo-colored image. B, emission spectrum from all pixels in A. C, image of photons arising from lanthanide centered emission. D, emission spectrum from all pixels in C. The green and red dashed lines in the spectra illustrate the spectral regions used for pseudocoloring.

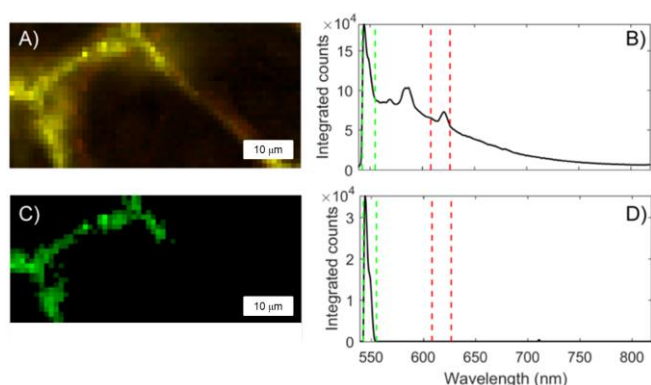


Figure 4. 30 by 60 micrometer images of a single region of CHO cells stained with terbium(III) acetate imaged in a confocal scanning microscope equipped with a CCD spectrometer and following 488 nm excitation. A, pseudocolour image. B, emission spectrum from all pixels in A. C, image of photons arising from lanthanide centered emission. D, emission spectrum from all pixels in C. The green and red dashed lines in the spectra illustrate the spectral regions used for pseudocoloring. For details on the image analysis, see SI.

We can now scrutinize the resolved lanthanide luminescence spectra from all pixels in all samples. As the spectra from all the europium(III) stained CHO cells are identical across all pixels and all images we can conclude that the geometry and donor atoms surrounding the europium ions are similar.^[1] The spectrum consists of narrow emission lines with a significant degree of fine structure. In the spectra obtained from the CHO cells, the 5D_0 to 7F_4 band at 690 nm shows a particular degree of fine structure that allows us to conclude that all the emitting europium(III) species are similar if not identical. This leads to a hypothesis where all the lanthanide ions predominantly bind to a single, specific structure in the CHO cell glycocalyx. Based on the studies performed using TEM and the fact that lanthanide

ions prefer hard anionic ligands,^[11a] we propose that these structures are anionic motifs in the glycocalyx. The binding pockets exhibited by the different anionic sugars are significantly different, indicating that the lanthanide ions must be bound either to hyaluronic acid, heparan sulfate, chondroitin sulfate or sialic acid residues. At this point we cannot differentiate which, but based on the relatively weak symmetry-dependent 580 nm band (the untreated spectra are available as supporting information) in the spectrum we can conclude that the europium(III) ions are located in identical, symmetric binding pockets.^[1]

To substantiate the claim that the lanthanide(III) ions are bound to a single structural motif of the glycocalyx, we produced control samples of europium(III) acetate with and in the absence of CHO cells. If either control gave rise to similar emission spectra, the claim could be disproved. Figure 5 shows the resolved emission spectra normalized to the 590 nm emission peak from samples with CHO cells stained with europium(III) along with spectra from control samples with europium(III) acetate dried in the formaldehyde with and without the SlowFade Gold mounting medium. Cursory inspection of Figure 5 shows that the shapes and peak ratios of the resolved spectra from the control samples are different from each other and from the spectrum from CHO cells. This confirms that the lanthanide ions are indeed bound to the CHO cell glycocalyx, not by the fixing or mounting agents.

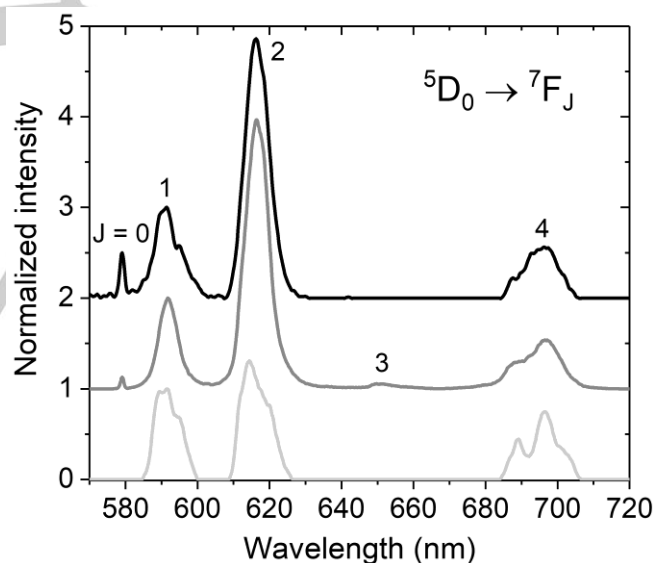


Figure 5 Europium(III) emission spectra from images recorded samples with europium(III) acetate in formaldehyde with (black) and without (grey) slow fade gold, and bound to the glycocalyx of CHO cells (light grey) and analyzed using the signal resolving algorithm. The spectra have been normalized to the 590 nm peak.

In summary, we have shown that it is possible to use direct excitation of terbium(III)- and europium(III) ion to obtain high contrast images of CHO cells. We used a home-built fluorescence microscope that allowed us to exploit the unique features of europium(III) luminescence to show that lanthanide(III) ions were present exclusively in the glycocalyx of

the CHO cells. The distinct europium(III) spectrum was used further, to show that the lanthanide ions all bind to similar ligands, which we suggest is one specific component of the glycocalyx. As we show that the highly inefficient direct excitation of low absorbance *f-f* transitions can be used for spectrally resolved fluorescence microscopy, we conclude that lanthanide luminescence following direct excitation should be accessible using conventional laser lines in standard fluorescent microscopes.

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Keywords: Lanthanide Luminescence • Spectroscopy • Coordination Chemistry • Microscopy • Glycocalyx

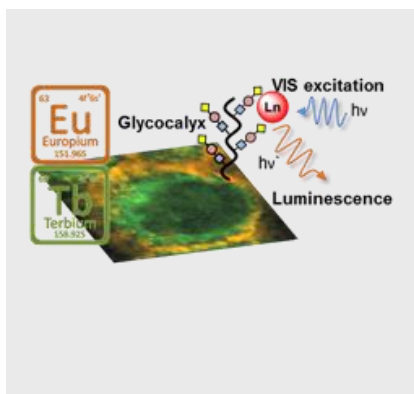
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Entry for the Table of Contents (Please choose one layout)

Layout 1:

COMMUNICATION

State-of-the-art microscopy allows us to record lanthanide luminescence spectra in every pixel when imaging cells. Here, we use this information to show that lanthanide(III) ions bind to a specific part of the glycocalyx of Chinese hamster Ovary or CHO cells.



Riikka Arppe-Tabbara, Miguel R. Carro-Temboury, Casper Hempel, Tom Vosch* and Thomas Just Sørensen**
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